



# *N*-glycosylation of human nicastrin is required for interaction with the lectins from the secretory pathway calnexin and ERGIC-53

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## Abstract

The  $\gamma$ -secretase complex, composed of four non-covalently bound transmembrane proteins Presenilin, Nicastrin (NCT), APH-1 and PEN-2, is responsible for the intramembranous cleavage of amyloid precursor protein (APP), Notch and several other type I transmembrane proteins.  $\gamma$ -Secretase cleavage of APP releases the A $\beta$  peptides, which form the amyloid plaques characteristic of Alzheimer's disease brains, and cleavage of Notch releases an intracellular signalling peptide that is critical for numerous developmental processes. NCT, a type I membrane protein, is the only protein within the complex that is glycosylated. The importance of these glycosylation sites is not fully understood. Here, we have observed that NCT *N*-linked oligosaccharides mediated specific interactions with the secretory pathway lectins calnexin and ERGIC-53. In order to investigate the role played by *N*-glycosylation, mutation of each site was performed. All hNCT mutants interacted with calnexin and ERGIC-53, indicating that the association was not mediated by any single *N*-glycosylation site. Moreover, the interaction with ERGIC-53 still occurred in PS1/2 double knockout cells as detected in immunoprecipitation as well as confocal immunofluorescence microscopy studies, which indicated that NCT interacted with ERGIC-53 prior to its association with the active  $\gamma$ -secretase complex.

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## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by two pathological hallmarks: the accumulation of senile plaques composed predominantly of amyloid- $\beta$  (A $\beta$ ) peptide and of neurofibrillary tangles (reviewed in [1]). The A $\beta$  peptide arises from the sequential cleavage of the amyloid precursor protein (APP), a type I transmembrane protein [2]. Two distinct enzymatic activities cleave APP giving rise to the A $\beta$  peptide. First, the  $\beta$ -site APP cleaving enzyme (BACE) cleaves APP, generating a C-terminal 99-amino acid long membrane anchored fragment termed C99. Subsequently, cleavage of C99 within its transmembrane domain (TMD) by the  $\gamma$ -secretase complex

generates different species of A $\beta$  peptide that differ slightly in length but vary greatly in their ability to aggregate, and an intracellular APP C-terminal domain [3,4]. In addition to APP, several other membrane proteins undergo regulated cleavage by  $\gamma$ -secretase, such as Notch, a cell surface receptor that is required for various cell fate decisions during embryogenesis [5].

The  $\gamma$ -secretase complex is a unique aspartyl protease whose active site appears to reside within the membrane. At present, the  $\gamma$ -secretase complex is composed by four noncovalently associated proteins: presenilin-1 (PS1) or its less abundant form presenilin-2 (PS2) [6], nicastrin (NCT) [7–10], PEN-2 and APH-1 [11,12]. Presenilin is believed to be the catalytic component of the complex [13–15], whereas NCT, PEN-2 and APH-1 were discovered more recently. NCT, a type I transmembrane protein, was isolated by biochemical co-purification with PS [10] and associates with the other  $\gamma$ -secretase components

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via the TM domain [16–18]. Furthermore, NCT is the only component of the  $\gamma$ -secretase complex that is glycosylated, though the roles played by the 16 potential *N*-linked oligosaccharides in NCT structure and function are not clear. Recently, it has been proposed that oligosaccharides may be involved in the initiation of the correct folding of some polypeptides in the ER [19]. Furthermore, proper folding of glycoproteins is facilitated in the ER by a battery of classical molecular chaperones (BiP, calnexin (CNX), calreticulin), and by proteins that catalyse protein disulfide-bond formation (PDI, Erp57). Altogether, they form a mechanism globally referred to as the “quality control” of protein folding [20]. Therefore, *N*-linked oligosaccharides do not only play a role in protein folding, but also have an active part in the quality control system [21].

Additionally, transport to subcellular compartments sometimes requires specialized pathways. After passing the CNX and calreticulin quality control cycle of the ER, glycoprotein transport from this compartment to the Golgi occurs, which involves the budding of COP-II coated vesicles from the ER that fuse with the ER-Golgi-Intermediate-Compartment (ERGIC), a subcellular structure that is composed of a multitude of tubulovesicular clusters in the vicinity of the *cis*-Golgi [22]. The lectin ERGIC-53, an unglycosylated type I transmembrane protein that cycles between the ER and the ERGIC [22,23], has properties of a mannose-selective lectin *in vivo* [24]. Lack of ERGIC-53 expression impairs secretion of a set of soluble glycoproteins, including blood coagulation factors V and VIII [25], and the cathepsin C precursor [26]. It has been suggested that the functional role of ERGIC-53 may be to operate as a molecular receptor for a specific subset of soluble, mannose-containing glycoproteins promoting their concentration in COPII vesicles followed by transport from the ER to the ERGIC.

In this study we have investigated the importance of the *N*-linked oligosaccharides present on human NCT (hNCT) for  $\gamma$ -secretase trafficking and activity. NCT specifically interacted with the lectins CNX and ERGIC-53. The interaction with ERGIC-53 was independent of PS1 association, and was not specific for any single *N*-glycosylation site. To the best of our knowledge, this is the first time that ERGIC-53 has been shown to associate with a membrane glycoprotein.

## 2. Materials and methods

### 2.1. DNA constructs

Expression vectors encoding for hNCT with a V5 epitope tag at the C-terminus (hNCT-V5) [18], human PS1 [27], APH-1a-HA and PEN-2-HA [28] have been previously described. Briefly, the cDNA encoding hNCT was a kind gift from Dr. Sangram Sisodia (University of Chicago, IL, USA) and was cloned into pcDNA3.1D/V5-His TOPO plasmid (Invitrogen) with the V5 tag epitope at the C-terminus of the protein. APH-1a and PEN-2 were cloned from a liver cDNA library (Clontech) and the resulting PCR products were cloned into pcDNA3.1D/V5-His TOPO plasmid (Invitrogen). The 3' HA epitope tag was introduced in-frame by PCR.

Single *N*-glycosylation sites mutants of hNCT (hNCT $\Delta$ ) were obtained by site-directed mutagenesis using QuickChange cloning techniques as

described by the manufacturer (Stratagene). Briefly, potential *N*-glycosylation sites with the known consensus sequence NXS/T were mutated to NXA. All hNCT and hNCT $\Delta$  constructs had a V5-tag epitope at the C-terminus of each protein for detection purposes. The identity of all plasmids and corresponding mutations were confirmed by sequencing analysis.

The plasmid encoding ERGIC-53 with a myc epitope tag introduced after the signal sequence (ERGIC-53-myc) [24] was a kind gift from Prof. H. P. Hauri (Biozentrum, University of Basel, Switzerland).

### 2.2. Cell culture and protein expression

NCT-deficient mouse embryonic fibroblasts (NCT<sup>-/-</sup>) and PS-1 and PS-2 deficient mouse embryonic stem cells (PS<sup>-/-</sup>) cells have been previously described [29,30]. Human embryonic kidney (HEK) 293T and NCT<sup>-/-</sup> cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose concentration (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin. ES cells, either wild-type (PS<sup>+/+</sup>) or deficient for PS1/2 (PS<sup>-/-</sup>), were grown in DMEM supplemented with 15% FBS, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100  $\mu$ M  $\beta$ -mercaptoethanol, 1% penicillin/streptomycin and 10<sup>5</sup> Units/l murine leukemia inhibitory factor (Chemicon). For transient expression, all cells were transfected with 2  $\mu$ g total DNA at approximately 70% confluency in 10 cm<sup>2</sup> dishes (approximately 1  $\times$  10<sup>6</sup> cell/dish) with Lipofectamine2000 (Invitrogen) according to the supplier's protocol. For hNCT expression in HEK293T cells, quadruple transfections with plasmids encoding for hPS1, hAPH-1-HA, hPEN-2-HA (0.5  $\mu$ g of each plasmid) were performed, and for the cases where ERGIC-53-myc was also expressed 0.4  $\mu$ g of each plasmid were used. For expression in NCT<sup>-/-</sup> MEF cells and PS<sup>-/-</sup> or PS<sup>+/+</sup> ES cells, double transfections with plasmids encoding for hNCT and ERGIC-53 (1  $\mu$ g of each plasmid) were performed. The plasmid encoding for green fluorescent protein (pTARGET-GFP), a non-related protein, was used to equalize the total amount of DNA transfected.

### 2.3. Antibodies

For immunoprecipitation studies the following antibodies were used: 7  $\mu$ l rabbit polyclonal #1712, which was produced by immunization of rabbits with purified recombinant thioredoxin-V5/His [31] for V5 tagged proteins, 5  $\mu$ l mouse monoclonal anti-myc tag (clone 9E10) for myc tagged proteins, 10  $\mu$ l goat polyclonal anti-CN-X-C20 raised against the C-terminus of human CNX (Santa Cruz Biotechnologies), and 3  $\mu$ l mouse monoclonal anti-PS1-NTF antibody raised against the NTF region of PS1 (antibody14); this antibody was a kind gift from Prof. Sam Gandy (Thomas Jefferson University, PA, USA).

For Western blot analysis, a mouse monoclonal anti-V5 epitope tag antibody (Invitrogen) was used for V5 epitope tagged protein visualization, and a polyclonal rabbit anti-NCT (Cell Signalling Technology) was used for endogenous NCT visualization.

### 2.4. Western blot analysis

For protein analysis, 24 h post-transfection, cell extracts were prepared in 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Roche). Cell lysates were incubated at 4 °C for 30 min, centrifuged at 10,000  $\times$  g for 10 min, and analyzed by SDS-PAGE with 10% acrylamide followed by Western blot analysis using horseradish peroxidase (HRP)-conjugated secondary antibody. Visualization was performed with ECL-Plus Western Blotting Detection Reagents (Amersham).

### 2.5. Co-immunoprecipitation

Co-immunoprecipitation assays were performed as previously described [28]. Briefly, cells (1  $\times$  10<sup>6</sup> cells) were lysed with 0.5 ml lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulfonate (CHAPSO), 5 mM EDTA and protease inhibitor cocktail. Pre-cleared lysates were incubated for 1 h at 4 °C with antibody previously coupled to protein A/G Plus agarose beads (Santa Cruz

Biotechnology). Immunoprecipitated proteins were eluted at 100 °C for 5 min with 20 µl sample buffer (0.08 M Tris–HCl pH 6.8, 2% sodium dodecylsulfate, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue). Samples were analyzed by Western blot as described above.

2.6. Glycosidase hydrolysis

Cell lysates were incubated with peptide: N-glycosidase F (PNGaseF) and endoglycosidase H (EndoH) according to supplier's protocol (New England Biolabs). Briefly, 30 µl of cell lysate (approximately 1.2×10<sup>5</sup> cell) were denatured in glycoprotein denaturing buffer at 100 °C for 10 min, and incubated with deglycosylation enzyme (PNGaseF or EndoH) in the corresponding buffer. Hydrolysis was carried out at 37 °C for 3 h. The samples were further analyzed by Western blot with anti-V5 antibody as described above.

2.7. Immunofluorescence microscopy

Murine PS<sup>+/+</sup> and PS<sup>-/-</sup> were grown on glass coverslips to half-confluence, and transfected with hNCT-V5 and ERGIC-53-myc. Twenty-four hours post-transfection, cells were washed with PBS containing 0.5 mM MgCl<sub>2</sub>, fixed with 4% (w/v) paraformaldehyde in PBS for 20 min and permeabilized with 0.1% (w/v) TX-100 in PBS for 15 min. Blocking was done with 1% BSA in PBS for 1 h and the coverslips incubated at room temperature for 2 and 1 h with primary and secondary antibodies, respectively. Antibody solutions were prepared in PBS containing 1% (w/v) BSA and washing was performed with PBS. Primary antibodies were rabbit anti-V5 epitope tag (1/500) [31], mouse anti-GM130 (1/200) (BD Bioscience) and mouse anti-myc (1/100); and secondary antibodies were donkey anti-mouse AlexaFluor-488 and donkey anti-rabbit AlexaFluor-594 (1/500) (Molecular Probes). Coverslips were mounted in Airvol and examined on a Leica Confocal (SP2+AOBS) microscope. For each picture, laser intensities and amplifier gains were adjusted in order to avoid pixel saturation. This was done using the GLOW LUT on the Leica Confocal Software. Each fluorophore used was excited and sequential detection was performed. Each picture consisted of a z-series of 20 images of 1024×1024 pixel resolution with a pinhole of 1.0 Airy unit. Colocalization analysis was performed using the open source Image J software version 1.30 (<http://www.rsbl.info.nih.gov/ij/>).

3. Results

3.1. hNCT associates with the lectins calnexin and ERGIC-53

For many glycoproteins, N-linked oligosaccharides play an active role in folding via association with CNX and calreticulin, chaperones that interact with newly synthesized glycoproteins in the ER [32,33]. We observed that hNCT<sup>wt</sup> associated with CNX through co-immunoprecipitation of CNX in HEK293T cells expressing hNCT<sup>wt</sup> followed by Western blot analysis (Fig. 1). Interestingly, comparison between the expression input levels and the co-immunoprecipitation data showed that the form of hNCT that associated with CNX (mgNCT in Fig. 1, second lane) in the Western blot analysis appeared at a molecular mass (MM) that was intermediate between the mature and immature forms of hNCT (mNCT and imNCT, respectively, in Fig. 1, first lane). The mgNCT form was sensitive to hydrolysis with EndoH (Fig. 1, third lane), and the product presented an apparent MM of approximately 80 kDa after hydrolysis. The hydrolysis results confirmed that mgNCT contained high-mannose type structures. This form of NCT probably corresponded to the monoglucosylated form, which has been reported in the case of other glycoproteins to have affinity for CNX [34].

In addition to CNX, other chaperones may also interact with newly synthesized glycoproteins. To investigate if ERGIC-53, a cargo receptor that mediates transport between the ER and ERGIC compartment through association with mannose residues present on glycoproteins, was capable of interacting with NCT, co-immunoprecipitation assays with HEK293T cells expressing a tagged version of ERGIC-53 (ERGIC-53-myc) and hNCT<sup>wt</sup> tagged with the V5 epitope (hNCT-V5) were performed. The results indicated that the immature form of hNCT associated with ERGIC-53 (Fig. 2A, third lane). Using higher exposure periods, we also observed that the mature form of hNCT was not co-immunoprecipitated with ERGIC-53 (data not shown).

In order to verify that the association between ERGIC-53 and hNCT was specific, a competition assay for the carbohydrate-binding site present on ERGIC-53 was also performed with methyl-α-D-mannopyranoside (αMM) (Fig. 2A, fourth lane). As expected, no hNCT binding was detected in the presence of αMM, indicating that this monosaccharide competed with the mannose containing oligosaccharides present on hNCT. Moreover, the same procedure performed in the presence of D- (+)-glucose showed that ERGIC-53 still interacted with hNCT (Fig. 2A, sixth lane), indicating that the disruption of the established association was mannose dependent. As control, we have observed that the presence of αMM did not disrupt γ-secretase complex association since hNCT could still be co-immunoprecipitated with hPS1 (Fig. 2B). Furthermore, there was no unspecific binding of hNCT to the protein A/G-agarose beads in the absence of anti-V5 antibody (Fig. 2C). Finally, the incubation with αMM did not inhibit immunoprecipitation using anti-V5 antibody (Fig. 2D). Together these results further supported that the interaction of ERGIC-53 with hNCT was specific.

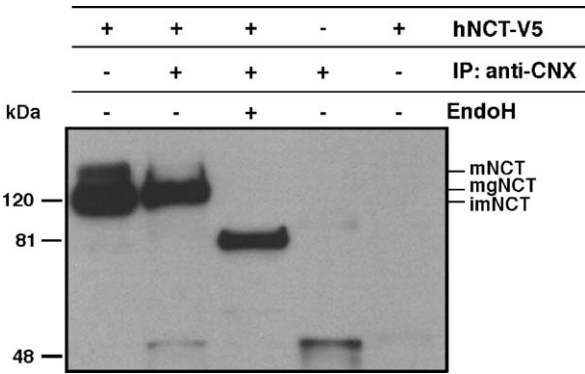


Fig. 1. hNCT associates with CNX. HEK293T cells transfected with hNCT-V5 (indicated above the figure) were lysed in 1% TX-100 lysis buffer and co-immunoprecipitated with a goat polyclonal anti-CNXX antibody (IP: anti-CNXX). Co-immunoprecipitation was performed with approximately 1×10<sup>6</sup> cells. The NCT form co-immunoprecipitated with CNX was treated with EndoH (third lane). As controls direct analysis of the cell lysate (approximately 2×10<sup>4</sup> cells) (first lane), co-immunoprecipitation where cell lysate was replaced by lysis buffer (fourth lane), and co-immunoprecipitation in the absence of antibody (fifth lane) were performed. Western blot was probed with mouse monoclonal anti-V5 tag antibody for hNCT detection. Mature NCT (mNCT), immature NCT (imNCT) and monoglucosylated NCT (mgNCT) are indicated in the figure.

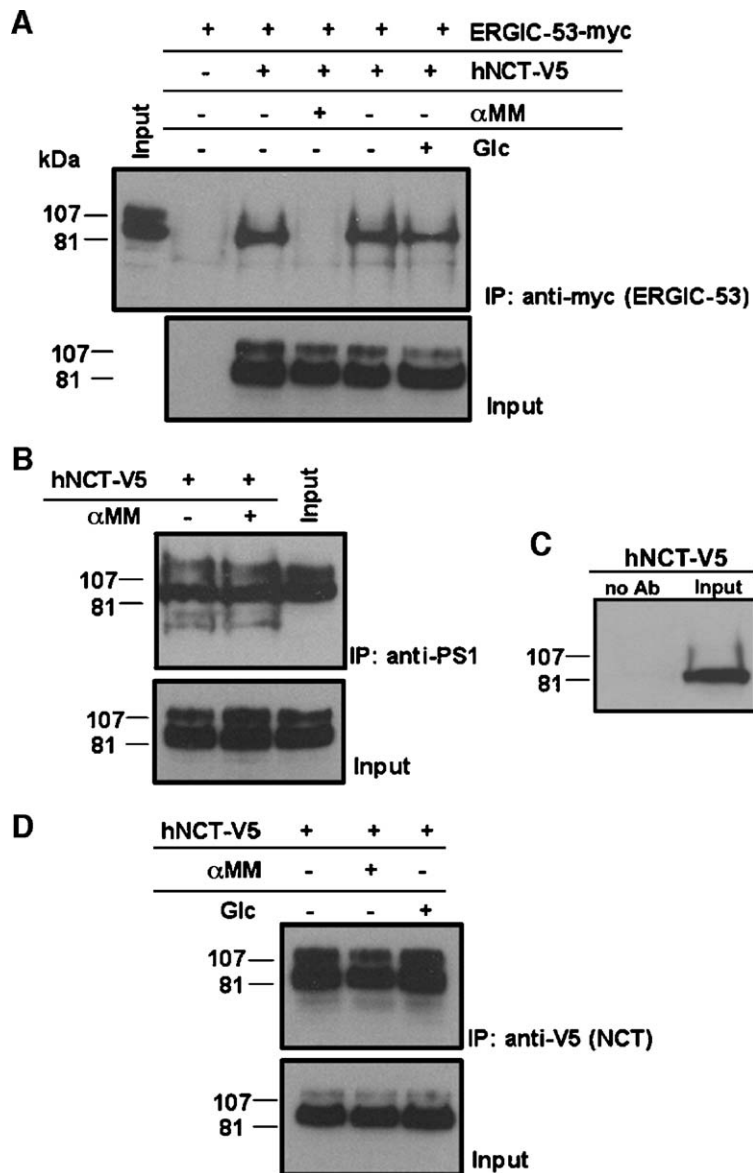


Fig. 2. hNCT associates with ERGIC-53. HEK293T cells, transfected with ERGIC-53-myc and hNCT-V5 (indicated above the Figure) were lysed in 1% CHAPSO and co-immunoprecipitated with a mouse monoclonal anti-myc antibody for ERGIC-53 (A), with a mouse monoclonal anti-PS1 antibody (B), and with a rabbit polyclonal anti-V5 antibody for hNCT-V5 (D). A competition assay was performed where 0.3 M methyl- $\alpha$ -D-mannopyranoside ( $\alpha$ MM),  $H_2O$ , or 0.3 M D-(+)-glucose (Glc) was added to the cell lysate before immunoprecipitation. Immunoprecipitates and corresponding cell lysates (Input) were analyzed by Western blot with a mouse monoclonal anti-V5 tag antibody to detect hNCT. An immunoprecipitation assay was performed in the absence of antibody (C, no Ab) to verify that co-immunoprecipitation results were not due to interaction of hNCT-V5 with protein-A/G agarose beads.

### 3.2. Generation and characterization of hNCT $\Delta$ mutants

To further characterize the role that *N*-glycosylation of NCT may play in mediating this interaction with CNX and ERGIC-53, site-directed mutagenesis was performed to mutate individual *N*-glycosylation sites in hNCT. Briefly, each mutant construct was obtained using the QuickChange cloning technique, where the potential *N*-glycosylation site was changed from NXS/T to NXA. The mutants are referred to as hNCT $\Delta$  followed by the amino acid number of the altered residue.

To assess whether the hNCT $\Delta$  constructs were transported properly, their glycosylation pattern was analysed after enzymatic deglycosylation with PNGaseF and EndoH. As expected,

hNCT $_{wt}$  appeared as both immature (imNCT, approximately 110 kDa) and mature (mNCT, approximately 130 kDa) form (Fig. 3). After hydrolysis with PNGaseF, which cleaves between the innermost GlcNAc and Asn residues of high mannose, hybrid and complex *N*-linked oligosaccharides, hNCT $_{wt}$  appeared with an apparent MM of approximately 80 kDa (Fig. 3)  $\blacktriangleleft$ , which corresponded to a totally deglycosylated form. In the presence of EndoH, which cleaves between the core GlcNAc-GlcNAc residues of high mannose and some hybrid *N*-linked oligosaccharides, hNCT $_{wt}$  appeared as two bands, one of which co-migrated with fully deglycosylated NCT representing protein in the ER and early Golgi (Fig. 3)  $\blacktriangleleft$ . The second band was partially resistant to EndoH at approximately 110 kDa (Fig. 3)  $\blacktriangleleft$ ,



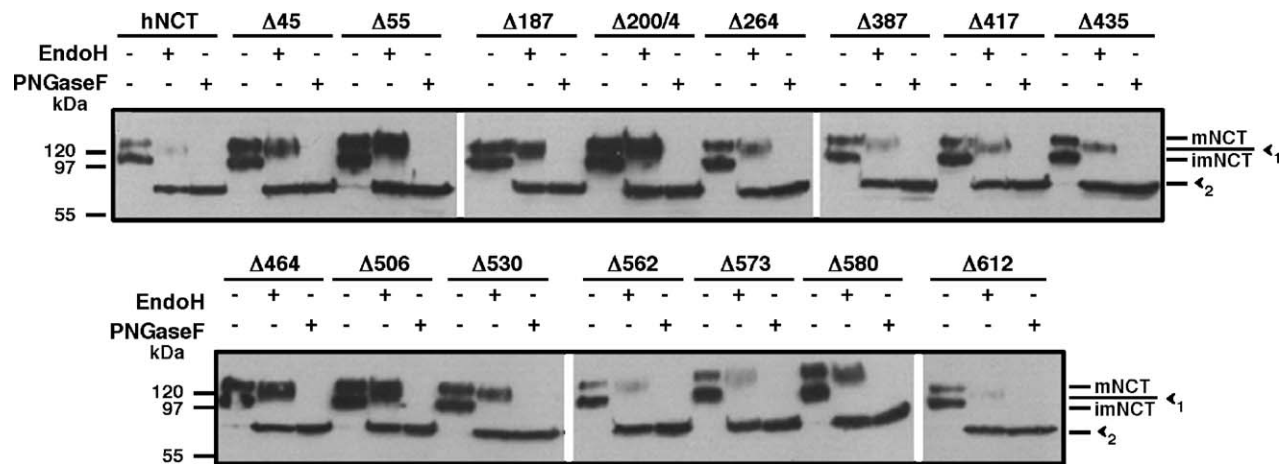


Fig. 3. Enzymatic deglycosylation of hNCT *N*-glycosylation mutants. HEK293T cells were transfected with hNCT or one of the fifteen hNCTΔ mutants, all containing a C-terminal V5 epitope tag for detection purposes. Cells were lysed in buffer containing 1% TX-100 and hydrolyzed with PNGaseF, EndoH or lysis buffer as a control. Western blot analysis using anti-V5 antibody was performed. The immature (imNCT) and mature (mNCT) forms of NCT can be visualized, as well as the EndoH resistant Golgi processed α<sub>1</sub> and the deglycosylated α<sub>2</sub> forms of hNCT.

consistent with mature NCT in which some but not all of the *N*-linked oligosaccharides have been processed in the Golgi to complex forms. The hNCTΔ mutants as well as NCT<sub>wt</sub> presented an immature EndoH sensitive ER form and a mature EndoH resistant Golgi form (Fig. 3). The mutation of a single *N*-glycosylation site did not allow us to verify if any of the mature hNCTΔ mutants were exhibiting a different EndoH resistance pattern. However, performing combined mutations of *N*-glycosylation sites could clarify this aspect. This experiment revealed that none of the single *N*-glycosylation site mutations disrupted transport and maturation of hNCT.

Previous studies have shown that an active γ-secretase complex requires the presence of a functional NCT for the endoproteolysis of PS1 [30] and processing of Notch [5] to occur. In order to determine if cleavage of PS1 and NotchΔE, a truncated form of Notch [35], was occurring in the presence of hNCTΔ mutants, NCT<sup>-/-</sup> cells were transfected with the indicated hNCTΔ mutants and with hPS1 or NotchΔE, respectively. Western blot analysis of cell lysates showed that hNCT<sub>wt</sub> and the hNCTΔ mutants were capable of mediating endopro-

teolysis of hPS1 and of cleaving NotchΔE (data not shown), indicating that no single *N*-glycosylation site deletion was sufficient to ablate γ-secretase activity. However, analysis of PS1-NTF produced and the amount of cleaved Notch product in the presence of each NCTΔ mutant showed an apparent reduction in γ-secretase activity when compared to hNCT<sub>wt</sub>. Further studies need to be performed in order to confirm this apparent reduction observed in activity. Ideally, functional assays should be performed in stable hNCTΔ mutant cell lines, or with an *in vitro* functional assay where hNCTΔ mutants were incorporated in the reconstituted γ-secretase activity.

3.3. Association of hNCTΔ mutants with lectins CNX and ERGIC-53

In order to understand which *N*-glycosylation site was mediating the association with lectins CNX and ERGIC-53, HEK293T cells expressing hNCTΔ constructs were co-immunoprecipitated with CNX (Fig. 4A). As expected, when taking into account the maturation pattern obtained for each

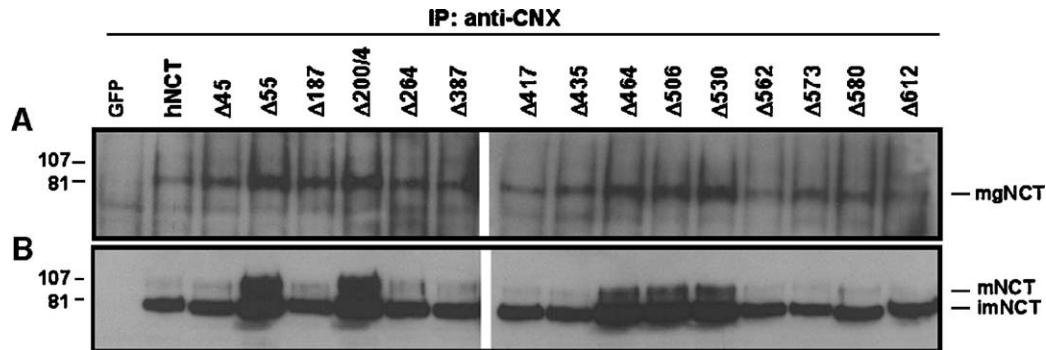


Fig. 4. Association of CNX with *N*-glycosylation mutants of NCT. HEK293T cells, endogenously expressing calnexin (CNX) and the indicated hNCTΔ mutants were lysed in buffer containing 1% CHAPSO and co-immunoprecipitated with an antibody to CNX. The immunoprecipitates were analyzed by Western blot with an anti-V5 antibody to detect co-immunoprecipitated hNCT and deletion mutants (A). Corresponding cell lysates were analyzed for hNCT and hNCTΔ mutant expression levels by Western blot against anti-V5 antibody (B). Both immature (imNCT), monoglucosylated NCT (mgNCT) and mature (mNCT) forms of NCT were indicated in the figure.

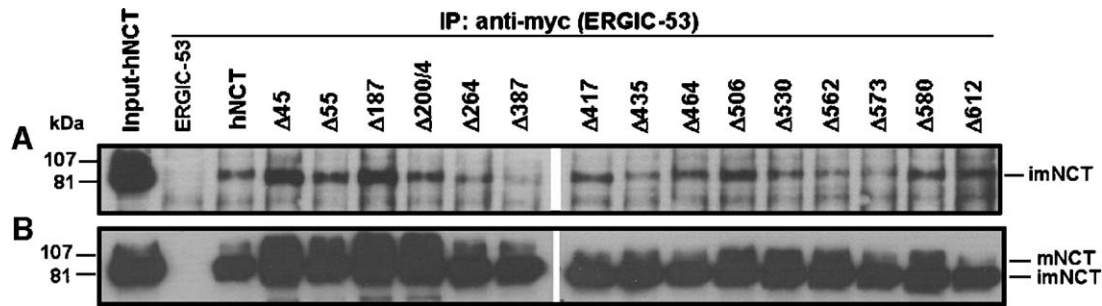


Fig. 5. Association of ERGIC-53 with *N*-glycosylation mutants of NCT. HEK293T cells, expressing ERGIC-53-myc and the indicated hNCTΔ mutants were lysed in buffer containing 1% CHAPSO and co-immunoprecipitated with an antibody to myc-epitope tag. The immunoprecipitates were analyzed by Western blot with an anti-V5 antibody to detect co-immunoprecipitated hNCT and deletion mutants (A). Corresponding cell lysates were analyzed for hNCT and hNCTΔ mutant expression levels by Western blot against anti-V5 antibody (B). Both immature (imNCT) and mature (mNCT) forms of NCT were indicated in the figure. Input-hNCT is a control-loading lane to facilitate hNCT band pattern visualization.

hNCTΔ mutant (Fig. 3), all hNCTΔ mutants were capable of interacting with CNX. This result indicated that all hNCTΔ mutants acquired a proper fold via association with CNX.

Additionally, hNCTΔ constructs and ERGIC-53 co-immunoprecipitation assays were performed in HEK293T cells expressing ERGIC-53-myc and hNCTΔ mutants. Despite the absence of specific *N*-linked oligosaccharide sites on hNCT, ERGIC-53 was capable of associating with each hNCTΔ mutant (Fig. 5A), indicating that this association was not mediated by any single *N*-glycosylation site. In all cases, comparison with non-immunoprecipitated hNCT (Fig. 5A, Input-hNCT) showed that only the immature forms of the hNCTΔ mutants were detected to interact with ERGIC-53, despite the presence of both immature and mature hNCT forms in the corresponding cell lysates (Fig. 5B). These results could be explained by the recognition specificity of ERGIC-53 towards high-mannose type oligosaccharides from *N*-glycosylated proteins [39], and to the higher amounts of immature hNCT relative to mature hNCT present in the extracts (Fig. 5B).

#### 3.4. NCT / ERGIC-53 association occurs in the absence of PS1

To determine if the presence of PS is required for the association between hNCT and the lectin ERGIC-53, co-immunoprecipitation assays with murine PS<sup>+/+</sup> and PS<sup>-/-</sup> cells expressing ERGIC-53-myc and hNCT-V5 were performed (Fig. 6A). The results showed that hNCT associated with ERGIC-53 independently of the presence of PS. Detection of endogenous NCT in PS<sup>+/+</sup> and PS<sup>-/-</sup> cells, showed that the presence of PS was required for the maturation of NCT and that probably only in the presence of PS did trafficking of NCT to the Golgi occur (Fig. 6B), as previously shown by other authors [29,36,37]. These findings were also verified by confocal microscopy where co-localization between ERGIC-53 and hNCT was observed in both murine PS<sup>+/+</sup> and PS<sup>-/-</sup> cells expressing ERGIC-53-myc and hNCT-V5 (Fig. 7). This indicated that the presence of PS is not a prerequisite for association between NCT and ERGIC-53 to occur. We further observed that ERGIC-53 was found in vesicles throughout the cytoplasm in PS<sup>+/+</sup> cells, however, it appeared in larger vesicles in the perinuclear area for the PS<sup>-/-</sup> cells. Therefore, the absence of PS appears to

interfere with the dynamics and morphology of the ERGIC compartment. In spite of this, co-localization of hNCT and ERGIC-53 was still observed.

Additional co-localization studies between hNCT-V5 and GM130, a *cis*-Golgi resident protein, were performed in both cell lines. We observed that in PS<sup>+/+</sup> cells the majority of hNCT co-localized with GM130, which indicated that it was transported to the Golgi compartment. On the other hand, in PS<sup>-/-</sup> cells co-localization with GM130 was very low, most of the fluorescence spread throughout the cytoplasm (Fig. 7). These results indicated that PS was required for hNCT trafficking to the Golgi compartment to occur.

Therefore, the immature NCT form, present in the PS<sup>-/-</sup> cells, was capable of associating with ERGIC-53. Furthermore,

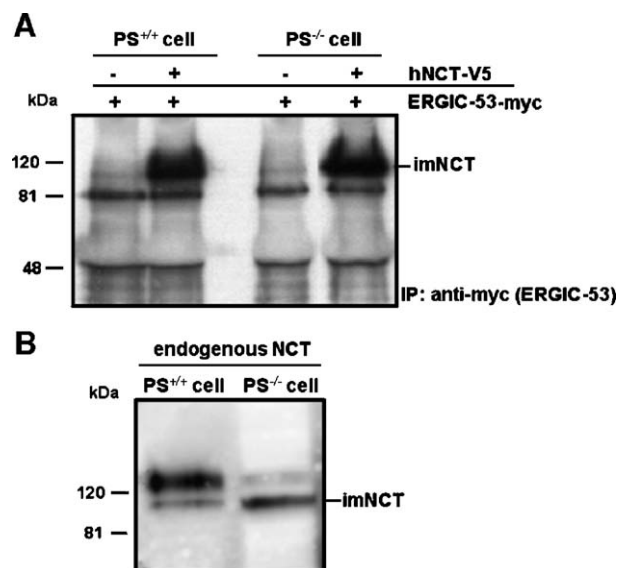


Fig. 6. hNCT/ERGIC-53 association occurs in the absence of PS1/2. Murine PS<sup>+/+</sup> and PS<sup>-/-</sup> cells, (A) expressing hNCT-V5 and ERGIC-53-myc, were lysed in buffer containing 1% CHAPSO and immunoprecipitated with an antibody to myc-epitope tag present on ERGIC-53. Immunoprecipitates were analysed by Western blot with anti-V5 tag antibody to detect hNCT. Western blot analysis of endogenous NCT (B) was performed in cell lysates prepared in buffer containing 1% TX-100 using a rabbit polyclonal anti-NCT antibody. Immature form of NCT (imNCT) was indicated in the figure.

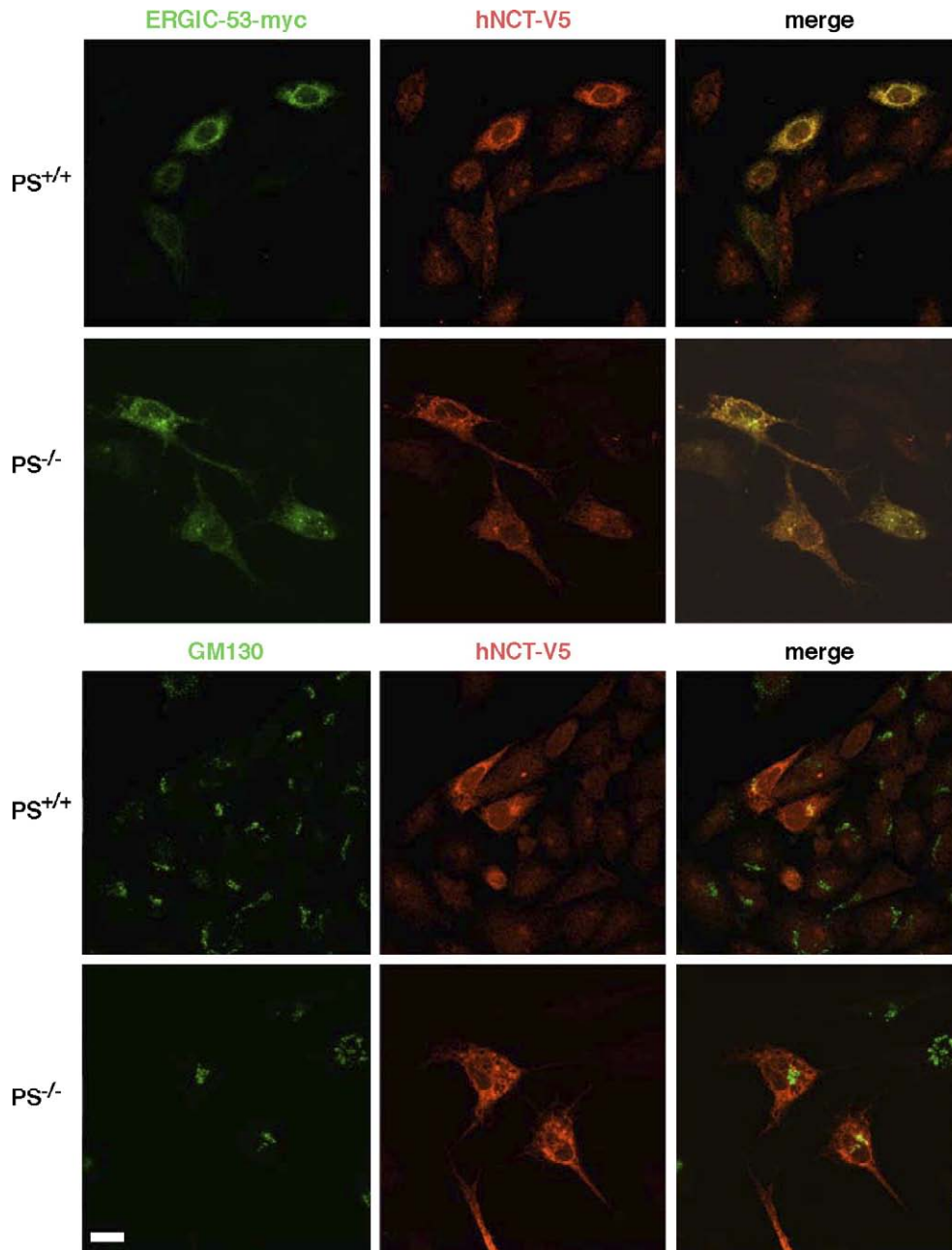


Fig. 7. Confocal immunofluorescence microscopy analysis of hNCT in PS<sup>+/+</sup> and PS<sup>-/-</sup> cells. Immunofluorescence microscopy was performed in murine PS<sup>+/+</sup> and PS<sup>-/-</sup> cells expressing hNCT-V5 and ERGIC-53-myc. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% TX-100 and processed for double labelling. Primary antibodies were the following: rabbit anti-V5 (hNCT-V5, red), mouse anti-GM130 (green) and mouse anti-myc (ERGIC-53-myc, green). Secondary antibodies were anti-rabbit coupled to Alexa 594 (red) and anti-mouse coupled to Alexa 488 (green). Merge panels show co-localization areas (yellow). Magnification: 630-fold, zoom 2. Scale bar: 10  $\mu$ m.

NCT associated with ERGIC-53 required the presence of PS to be transported to the Golgi.

#### 4. Discussion

NCT is one of the four components identified as part of the  $\gamma$ -secretase complex, an unusual membrane-bound aspartyl protease with the ability to cleave certain substrate proteins at peptide bonds buried within the hydrophobic environment of the lipid bilayer. NCT, a type I transmembrane glycoprotein

with 16 potential *N*-glycosylation sites, has been shown to contain a mixture of high-mannose and complex type oligosaccharides. The importance of this extensive carbohydrate array for NCT function is not fully understood. To investigate more carefully the role of NCT folding and how this regulates its transport from the ER, we investigated the interaction of hNCT with lectin-like molecular chaperones from the quality control/secretory pathway: CNX [38] and ERGIC-53 [22]. We observed that hNCT<sup>wt</sup> strongly interacted with CNX, which indicated that this chaperone participated in



the folding of hNCT. This interaction indicates that hNCT probably uses this quality control pathway to acquire a proper fold. Since the ectodomain of Nicastrin has been implicated in the recognition of  $\gamma$ -secretase substrates [40], it is probable that the interaction hNCT/CNX is important to promote a proper conformation of hNCT ectodomain, which is essential for substrate recognition.

*N*-linked oligosaccharides not only have a functional role in protein folding, but in some cases, are also involved in transport and targeting of glycoproteins. ERGIC-53, a lectin transport receptor, recognizes folded, high-mannose type glycoproteins in the ER [39] after they have been released from the quality control machinery for glycoprotein folding (calnexin/calreticulin) and subjected to glucose trimming [32]. In this study we have shown that ERGIC-53 specifically interacted with hNCT, and that the presence of  $\alpha$ MM disrupted this association, indicating that this interaction was carbohydrate specific, and involved high-mannose type oligosaccharides from hNCT and the carbohydrate recognition domain (CRD) from ERGIC-53.

To determine if any specific *N*-linked glycosylation sites were important for NCT interaction with CNX and ERGIC-53, we produced single site deletion mutants (hNCT $\Delta$ ) lacking each of the *N*-linked addition sites in turn. Association of hNCT $\Delta$  mutants with CNX and ERGIC-53 still occurred, indicating that these mutants were acquiring a proper fold via the CNX quality control pathway and that hNCT had more than one site containing high mannose type oligosaccharides. Furthermore, association between NCT/ERGIC-53 was not specific for a single site. At this point, the benefit of the NCT/ERGIC-53 association is not fully understood. A likely scenario is that ERGIC-53 may act as a transport chaperone facilitating ER to ERGIC transport of the  $\gamma$ -secretase complex, or that to some extent ERGIC-53 may stabilize the  $\gamma$ -secretase complex in the ER and thereby promote optimal assembly of NCT with other  $\gamma$ -secretase complex components.

Another interesting feature about this complex is that the presence of each  $\gamma$ -secretase component influences the targeting/maturation of the other components. In the present work we have observed that the mature form of NCT is largely or completely absent in murine PS<sup>-/-</sup> cells and that trafficking of NCT to the Golgi does not occur in these cells, with only the immature EndoH-sensitive NCT band being observed, as has been reported by others [29,36,37]. Despite the absence of PS1/2, NCT was still capable of associating with ERGIC-53. An ER-retention signal has been found to exist on the C-terminus of PS that could prevent unassembled subunits from leaving the ER, but that is masked upon full assembly [41]. It is also possible that NCT could contain an ER-retention signal that would be masked upon association with other members of the  $\gamma$ -secretase complex. In such a model, one could postulate that NCT would interact with ERGIC-53 in the ER and that transport of the  $\gamma$ -secretase complex via this cargo receptor lectin would occur after association of NCT with PS. Further studies need to be performed to understand in more detail the importance of the  $\gamma$ -secretase complex requiring the cargo receptor lectin ERGIC-53 for transport/targeting to the ERGIC/*cis*-Golgi compartment.

In summary, NCT interacts with secretory pathway lectins CNX and ERGIC-53. *N*-glycosylation sites influence the proper folding of NCT on one hand, and seem to have a role in ER-assembly and ER-exit of NCT, on the other hand.

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